



University of Groningen

Transformation of Cowpea *Vigna unguiculata* with a Full-Length DNA Copy of Cowpea Mosaic Virus M-RNA

Hille, Jacques; Goldbach, Rob

Published in:
Plant Science

DOI:
[10.1016/0168-9452\(87\)90135-X](https://doi.org/10.1016/0168-9452(87)90135-X)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1987

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Hille, J., & Goldbach, R. (1987). Transformation of Cowpea *Vigna unguiculata* with a Full-Length DNA Copy of Cowpea Mosaic Virus M-RNA. *Plant Science*, 48(2). [https://doi.org/10.1016/0168-9452\(87\)90135-X](https://doi.org/10.1016/0168-9452(87)90135-X)

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

TRANSFORMATION OF COWPEA *VIGNA UNGUICULATA* WITH A FULL-LENGTH DNA COPY OF COWPEA MOSAIC VIRUS M-RNA

JUAN A. GARCIA*, JACQUES HILLE, PIETER VOS and ROB GOLDBACH^a

Departments of Molecular Biology and ^aVirology, Agricultural University, De Dreijen 11, 6703 BC Wageningen (The Netherlands)

(Received June 7th, 1986)

(Revision received September 6th, 1986)

(Accepted September 6th, 1986)

A full-length DNA copy of the M-RNA of cowpea mosaic virus (CPMV), supplied with either the 35S promoter from cauliflower mosaic virus (CaMV) or the nopaline synthase promoter from *Agrobacterium tumefaciens*, was introduced into the T-DNA region of a Ti-plasmid-derived gene vector and transferred to cowpea *Vigna unguiculata* cells. Southern blot analysis of the transformed callus tissue obtained confirmed the integration of the viral DNA copy into the plant DNA. Northern blot analysis revealed that full-length transcripts of more than 3500 nucleotides long were produced from the integrated copy supplied with either promoter, the CaMV 35S promoter being approx. 10-fold more active than the nopaline synthase promoter. The production of full-length M-RNA-like transcripts in transformed cowpea calli will permit to study M-RNA-expression in the absence of B-RNA and the infectivity of cloned viral DNA copies.

Key words: cowpea mosaic virus; *Vigna unguiculata*; viral DNA copy; plant transformation

Introduction

Cowpea mosaic virus (CPMV) has a genome consisting of a large (B) and a small (M) RNA segment, that are separately encapsidated. The genomic RNAs are characterized by a small protein (denoted VPg) covalently linked to the 5'-end and a poly(A) tail at the 3'-end (for review see Ref. 1). The expression of both RNAs involves the production of large polyproteins from which the smaller, functional proteins are generated by proteolytic cleavages [2–4].

Determination of the nucleotide sequence of M-RNA (comprising 3481 nucleotides exclusive of the poly(A) tail) reveals the presence of a unique long, open reading

frame in this genome segment [5]. Upon in vitro translation M-RNA produces two overlapping polyproteins of ~105 000 (105K) and ~95 000 (95K) which are the result of two in-phase AUG initiation codons (6–8 and Fig. 1). Proteolytic cleavage of these polyproteins generates cleavage products of 60K, 58K and 48K, of which the 60K product represent a direct precursor to the two capsid proteins VP37 and VP23 (Fig. 1). The function of the 58K and 48K proteins has remained unknown so far while, moreover, the use of both initiation sites on M-RNA still has to be verified in vivo.

To study the genetic properties of M-RNA we decided to supply a cloned, full-length DNA copy of this RNA with transcription regulatory sequences active in plants and to transform cowpea (*Vigna unguiculata*) cells with such constructs using a disarmed *Agrobacterium* Ti-plasmid-derived vector. In this way, continuous expression of this copy would be accomplished allowing study of

*Present address: Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Cantoblanco 28049, Madrid, Spain.

Abbreviations: CaMV, cauliflower mosaic virus; CPMV, cowpea mosaic virus; kb, kilo base pairs.

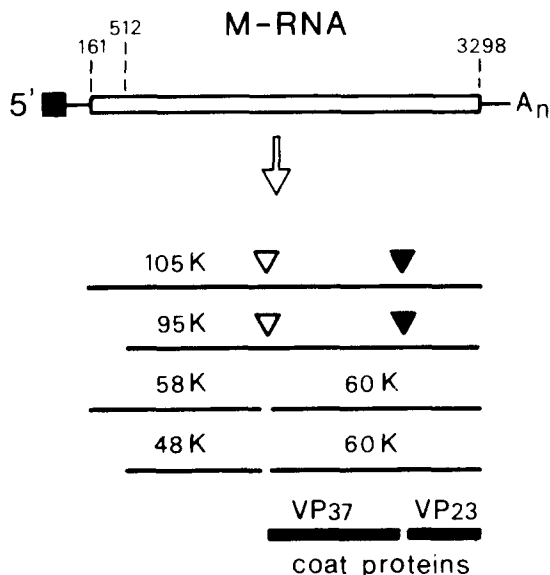


Fig. 1. Model for the translation of CPMV M-RNA. The scheme is largely based on *in vitro* translation studies. The positions of potential start (161, 512) and stop (3298) codons are indicated. In both wheat germ extract and rabbit reticulocyte lysate M-RNA is translated into two primary translation products of ~105K and ~95K which are proteolytically cleaved by two different B-RNA-encoded proteases to give the mature capsid proteins VP37 and VP23 together with two products of 58K and 48K. Symbols: ■, VPg; ▽, glutamine-methionine cleavage site; ▴, glutamine-glycine cleavage site.

the expression of M-RNA in uninfected host cells and facilitating the achievement of infectious transcripts from such copies, which would offer the possibility to study the genetic properties of the viral RNA genome by site-directed mutagenesis on DNA level. Although for cowpea, like for most leguminous plants, no regeneration procedure is currently available, the production of transformed cowpea callus using *A. tumefaciens*-mediated gene transfer has recently been described by us [9]. Following this approach we now report the stable introduction of a DNA copy of M-RNA in cowpea cells and the subsequent production of transformed cowpea callus in which this copy is expressed. The results reveal, moreover, that the 35S promoter

of cauliflower mosaic virus is approximately 10-fold more active in cowpea cells than the nopaline synthase promoter of *Agrobacterium*.

Materials and methods

Bacterial strains and plasmids

The strains MH1 (a gift from Dr. L. van der Eb, Leiden) and HB101 [10] were used as *Escherichia coli* hosts. The *E. coli* strain GJ23 containing the plasmid R64drd 11 and pGJ28 [11] supplied *tra* and *mob* functions in conjugation experiments. The *A. tumefaciens* strain C58CI harboring plasmid pGV3850 [12], pGV3850 :: 1103neo(dim) (a gift of Dr. L. Herrera-Estrella, see also Ref. 13) or its recombination derivatives was used for transformation of cowpea *V. unguiculata*. The plasmids pBR322 [14], pCa305 [15], pNop6 [16] and pCaMV35S [17] have been described previously. Plasmids pCM302 (P. Vos, unpublished) is a derivative from pCM423 [8] containing a poly(A) track of 50 nucleotides long.

DNA preparation

Plasmid DNA was prepared by the alkaline lysis method as described [18]. Total DNA from *A. tumefaciens* containing Ti-plasmid was isolated according to Ooms et al. [19] and plant DNA following the CTAB procedure of Murray and Thomson [20]. Digestion with restriction endonucleases of plasmid DNA was performed according to the supplier's instructions and with 20 units/μg for total bacterial or plant DNA. Conditions used for ligation of DNA fragments and subsequent transformation of competent *E. coli* cells have been described previously [5]. DNA fragments used for ligation were purified by extraction from low-melting agarose [21].

Bacterial conjugation

Conjugation involving *E. coli* and *A. tumefaciens* strains were performed as described by Van Haute et al. [11].

Transformation of cowpea

For transformation of cowpea *V. unguiculata* cells we used the leaf disc procedure described by Horsch et al. [22] with some modifications. Discs punched from primary leaves of 6-day-old cowpea plants were submerged for 2 min in a 1:10 dilution of an overnight culture of *A. tumefaciens* containing a Ti-plasmid derivative and blotted dry on filter paper. After inoculation the discs were incubated at 25°C on nurse culture plates containing callus-inducing GS3 medium [23] solidified with 0.8% agar, a feeder layer of *Petunia* 'commanche-albino' cells and a sheet of Whatmann 5 filter paper. After 2 days the discs were transferred to petri dishes with GS3 medium containing cefotaxime and vancomycin (200 µg/ml of each) to stop bacterial growth. As soon as distinct development of callus tissue was observed (about 10 days later) the discs were transferred to petri dishes containing GS3 medium containing antibiotic G418 (50 µg/ml) to select transformed plant cells. The individual calli obtained were subcultured every 20 days. After the first three transfers cefotaxime and vancomycin were omitted from the medium and kanamycin (100 µg/ml) instead of G418 was used to maintain selective pressure.

Southern blot analysis

DNA fragments originating from digestion of total plant DNA (5 µg) or total *A. tumefaciens* DNA containing Ti-plasmid (0.5 µg) with restriction endonucleases were separated by electrophoresis in 1% agarose gels, transferred to nitrocellulose paper and hybridized to ³²P-labeled probes as described [9].

Poly(A)⁺ RNA isolation and Northern blot analysis

Frozen cowpea calli (approx. 10 g) were ground to a fine powder under liquid nitrogen. Twenty milliliters of a hot (85°C) 1:1 mixture of 0.1 M lithium chloride, 1% sodium dodecyl sulphate, 10 mM EDTA and distilled phenol was added and the suspension vigorously shaken for 30 min at room temperature.

After addition of 10 ml of chloroform the mixture was shaken for another 10 min. After centrifugation at 15 000 × *g* for 30 min the aqueous phase was removed, re-extracted with chloroform and made 2 M in lithium chloride. RNA was precipitated overnight at 4°C, and subsequently collected by centrifugation, washed once with 2 M lithium chloride and twice with 80% ethanol, dried in vacuo and dissolved in 10 mM Tris-HCl (pH 7.5), 0.5 mM EDTA. Poly(A)⁺ RNA purified by oligo(dT) cellulose and separated in a 1% agarose gel containing formaldehyde [18] was subsequently transferred to nitrocellulose paper and hybridized to ³²P-labeled DNA probes as described by Thomas [24].

Results

Introduction of a DNA copy of CPMV M-RNA in the T-region of pGV3850 :: 1103-neo(dim)

In a previous report we have described the successful, *Agrobacterium*-mediated transfer of a chimaeric kanamycin resistance gene to cowpea cells and the subsequent production of kanamycin-resistant callus tissue [9]. This was established by inoculating cowpea leaf discs with an *A. tumefaciens* strain harboring the non-oncogenic Ti-plasmid derivative pGV3850 :: 1103-neo(dim) [13] in which the T-DNA sequences were replaced by two copies of a chimaeric kanamycin resistance gene and pBR322 sequences (Fig. 2). The same approach has now been followed to introduce a transcriptionally active full-length DNA copy of CPMV M-RNA (3481 bases long, exclusive of the poly(A) tail) into the chromosomal DNA of cowpea. For this purpose a cloned, full-length DNA copy of M-RNA (pCM302), previously described and used for in vitro expression studies [8], was first supplied with either the *Agrobacterium* nopaline synthase (nos) promoter [25] at one hand and with the nos terminator sequence at the other (see Fig. 2). Plasmid pNop6 [16] was used to place this DNA

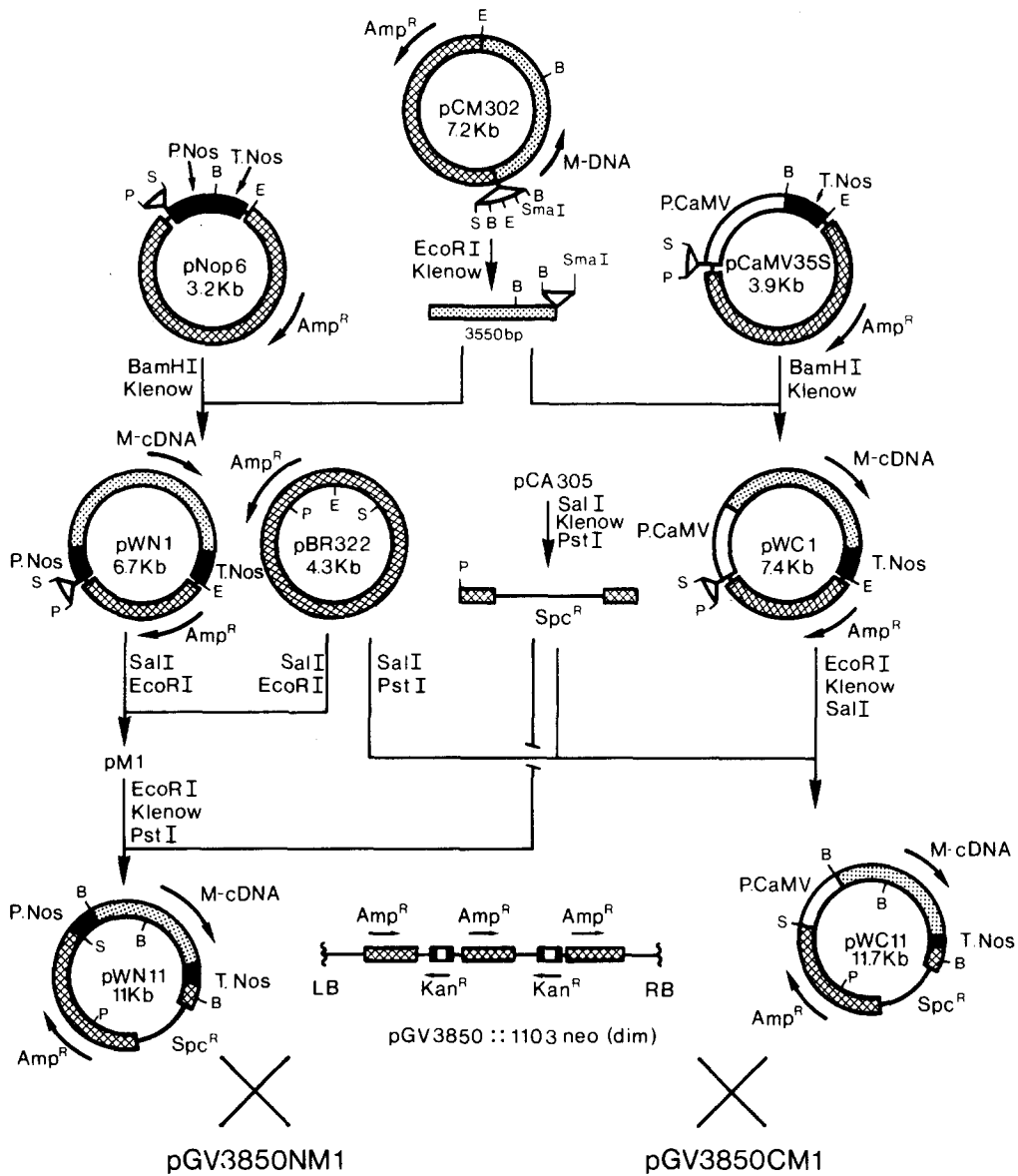


Fig. 2. Construction of plasmids pGV3850NM1 and pGV3850CM1. From plasmid pCM302 the *EcoRI* fragment containing the complete DNA copy of M-RNA was isolated, made blunt end and ligated to Klenow-treated *Bam*HI-digested pNop6 (containing nopaline synthase promoter and polyadenylation signals) or pCaMV35S (containing the CaMV 35S promoter sequence and the nopaline synthase polyadenylation signal), resulting in plasmids pWN1 and PWC1, respectively. Plasmid pM1 was obtained by cohesive ends ligation of the *EcoRI*-*Sal*I fragment of pWN1, containing the viral cDNA sequence, to the *EcoRI*-*Sal*I fragment of pBR322 containing the ampicilline resistance gene and the origin of replication. Plasmid pWN11 was obtained by ligating a *Pst*I-*Sal*I fragment (*Sal*I-end previously filled with Klenow enzyme) of pCA305, containing a gene for spectinomycin resistance (Spc^R), to a *Pst*I-*Eco*RI fragment (*Eco*RI-end previously filled with Klenow enzyme) of pM1. Plasmid pWC11 was obtained by three-points ligation of the *Sal*I-*Eco*RI fragment (*Eco*RI end filled with Klenow enzyme) from pWC1 containing the viral cDNA sequence, the *Pst*I-*Sal*I fragment (*Sal*I end filled with Klenow enzyme) of pCA305 containing the spectinomycin resistance gene, and the *Sal*I-*Pst*I fragment of pBR322 containing the origin of replication, respectively. Plasmids pWN11 and pWC11 were transferred to *A. tumefaciens* containing plasmid pGV3850 :: 1103neo(dim) by conjugation using the helper plasmids pGJ28 and R64did11. By subsequent single cross-overs between regions of homology within the T-region of plasmid pGV3850 :: 1103neo(dim) and pWN11 or pWC11 the resulting plasmids pGV3850NM1 and pGV3850CM1 were obtained, respectively. B, E, P and S are restriction enzymes *Bam*HI, *Eco*RI, *Pst*I and *Sal*I, respectively.

copy, excised with *Eco*RI from pCM302, under the control of nos promoter and nos terminator sequences, while the *bom* site

of pBR322 and the spectinomycin resistance (Spc^R) gene from pCA305 [15] were introduced following the protocol depicted in

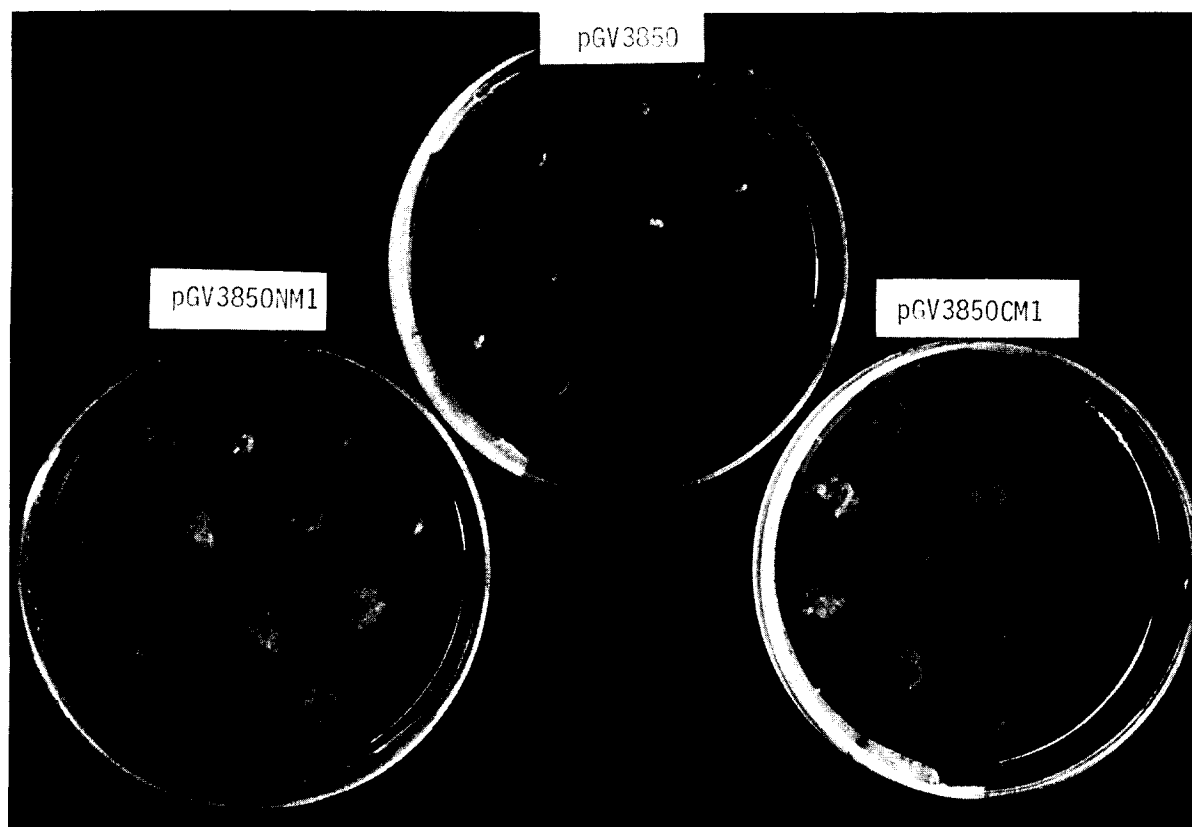


Fig. 3. Cowpea *V. unguiculata* calli transformed with *A. tumefaciens* carrying plasmids pGV3850 [12], pGV 3850NM1 or pGV3850CM1 grown in GS3 medium containing 50 μ g/ml antibiotic G418.

Fig. 2, resulting in plasmid pWN11. A similar construct (pWC11) was obtained in which the M-RNA copy was placed under the control of the CaMV 35S promoter, employing pCaMV35S [17] instead of pNop6.

The complete sequences of pWN11 or pWC11 were next introduced into the T-region of pGV3850 :: 1103neo(dim) by homologous recombination with the pBR322

sequences present in this region. *Agrobacterium* cells carrying the desired cointegrates pGV3850NM1 or pGV3850CM1 (containing the DNA copy of M-RNA supplied with either the nos or the CaMV 35S promoter, respectively, see Fig. 2) were selected on medium containing spectinomycin. Co-integration was verified by Southern hybridization (data not shown).

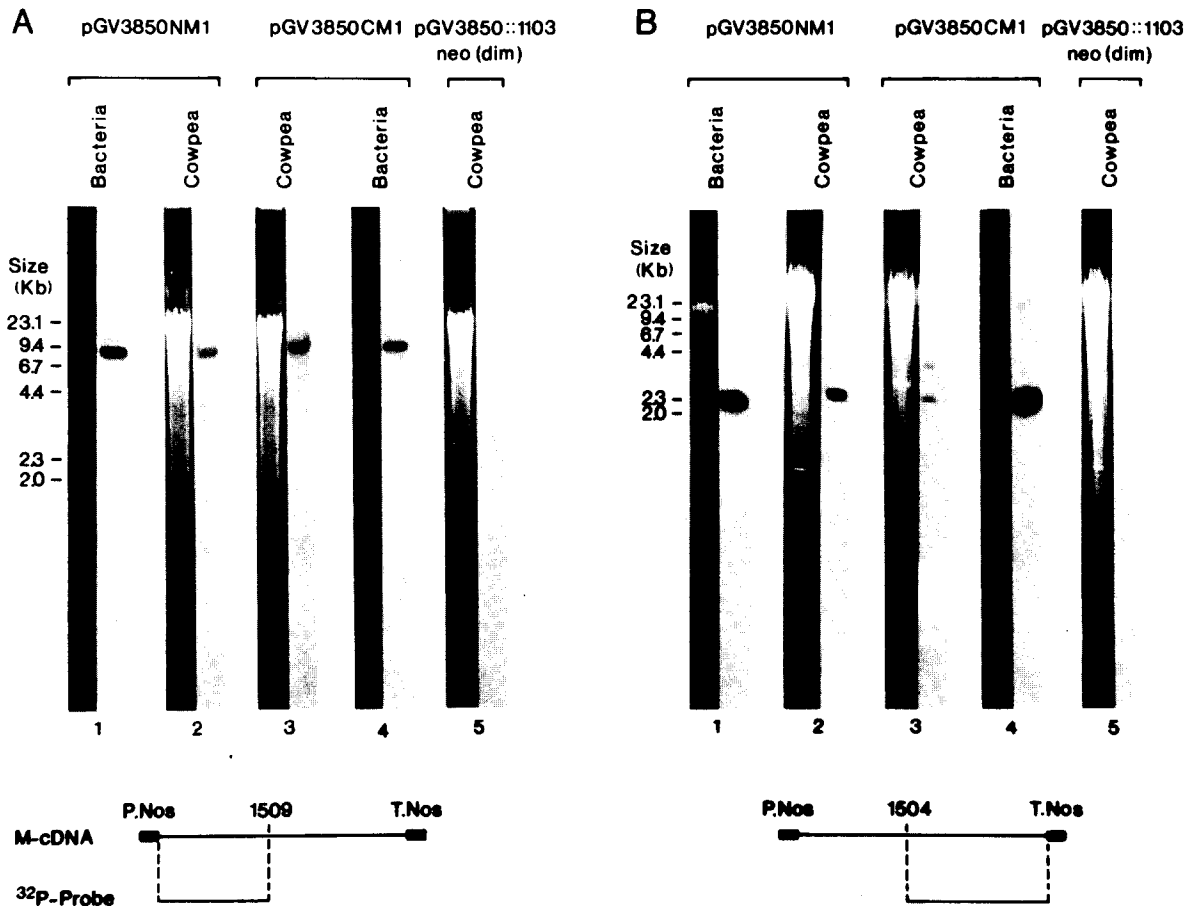


Fig. 4. Southern blot analysis of DNA from transformed cowpea calli. DNA isolated from cowpea callus tissue transformed with *A. tumefaciens* carrying plasmids pGV3850NM1, pGV3850CM1 or pGV3850 :: 1103neo(dim) and DNA from bacteria containing these plasmids were digested with both *SalI* and *PstI* (panel A) or with *BamHI* alone (panel B). The resulting fragments were separated in 1% agarose gels, blotted onto nitrocellulose filters and subsequently hybridized to ³²P-labeled probes containing M-RNA-specific sequences. The two probes used in panel A and B, respectively, were synthesized using single-stranded DNA from M13-derived phages as template. In both cases the template phage DNA contained positive-strand sequences of M-RNA, the regions comprised being indicated at the bottom of each panel. Each lane contains at the left the ethidium bromide-stained gel and at the right the autoradiograph of the nitrocellulose blot after hybridization. The position and size of the fragments generated by *HindIII*-digestion of phage λ DNA are shown at the left of each panel.

Transformation of cowpea cells using Ti-plasmids pGV3850NM1 and pGV3850CM1

To transfer and integrate the T-regions of pGV3850CM1 and pGV3850NM1 into cowpea chromosomal DNA we employed the leaf disc transformation method previously described [9,22]. Transformant calli were selected on GS3 medium [23] containing antibiotic G418 (50 µg/ml). Hence, transformation using pGV3850CM1 or pGV3850NM1 resulted in both cases in the development of cowpea calli able to grow in the presence of G418 or kanamycin while control (transformed using pGV3850) calli were not (Fig. 3). Resistant calli indeed contained nopaline synthase activity (data not shown), providing a first indication that these tissues were actually transformed.

Genomic analysis of kanamycin-resistant calli

To verify the integration of the M cDNA sequences into the genomic DNA of the resistant calli, DNA samples extracted from such tissues were analyzed by Southern blotting hybridization using probes containing M cDNA sequences. Figure 4 shows such analysis for DNA prepared from cowpea calli transformed using pGV3850NM1 or pGV3850CM1 and subsequently digested with restriction enzymes which cut at known places in the T-region of the plasmids employed. Digestion of transformed callus DNA with *Pst*I and *Sal*I, and subsequent hybridization using a radiolabeled probe containing cloned viral cDNA comprising the M-RNA sequence from the 5'-end to nucleotide position 1509, revealed a single hybridizing fragment with the expected size of 8.1 or 8.7 kilo base pairs (kb), respectively, when pGV3850NM1 or pGV3850CM1 was used for transformation (Fig. 4A, lanes 2 and 3). These fragments, which were absent in the digests of control calli (Fig. 4A, lane 5), comigrated with the single hybridizing bands in the *Pst*I/*Sal*I digests of the two vector construction used (Fig. 4A, lanes 1 and 4). Similarly, digestion of DNA

from transformed calli with *Bam*HI and hybridization with a cDNA probe comprising the viral sequence from nucleotide position 1504 downstreams to the 3'-end, resulted in a hybridizing band of ~2.5 kb that comigrated with the single, reacting *Bam*HI fragment from the transformation vectors used (c.f. lanes 1–4 in Fig. 4B). The extra fragment of ~4 kb in the *Bam*HI digest of callus transformed using pGV3850CM1 probably represents a partial digestion product. Hence, the hybridization patterns obtained reveal that integration of the complete viral cDNA sequence had occurred without any significant deletion or rearrangement.

Transcriptional analysis of integrated copies of M-RNA

Transcriptional activity of the integrated copies of CPMV M-RNA was determined by Northern analysis of poly(A)⁺ RNA using cloned cDNA sequences of M-RNA as probe (Fig. 5). The poly(A)⁺ RNA fraction of both callus tissue transformed using pGV3850NM1 (i.e. M-RNA copy supplied with nos promoter) and callus tissue transformed using pGV3850CM1 (M-RNA copy supplied with the CaMV 35S promoter) contained transcripts derived from the integrated viral copies (Fig. 5, lanes 3 and 4). In pGV3850NM1-transformed calli a single hybridizing transcript was detected approximately comigrating with natural M-RNA extracted from virions (c.f. lanes 1 and 3 in Fig. 5). The expected size of the transcript was approximately 3750 bases, composed of ~35 bases from the nos leader sequence, 3531 bases from the viral cDNA sequence, and ~200 bases of nos sequence at the 3'-end of the gene. Instead, in pGV3850CM1-transformed calli two large transcripts were detected, one similar in size to M-RNA or slightly larger and the other significantly shorter than natural M-RNA. The largest of these RNA species probably represented the intact, full-length transcript of the integrated M-RNA copy

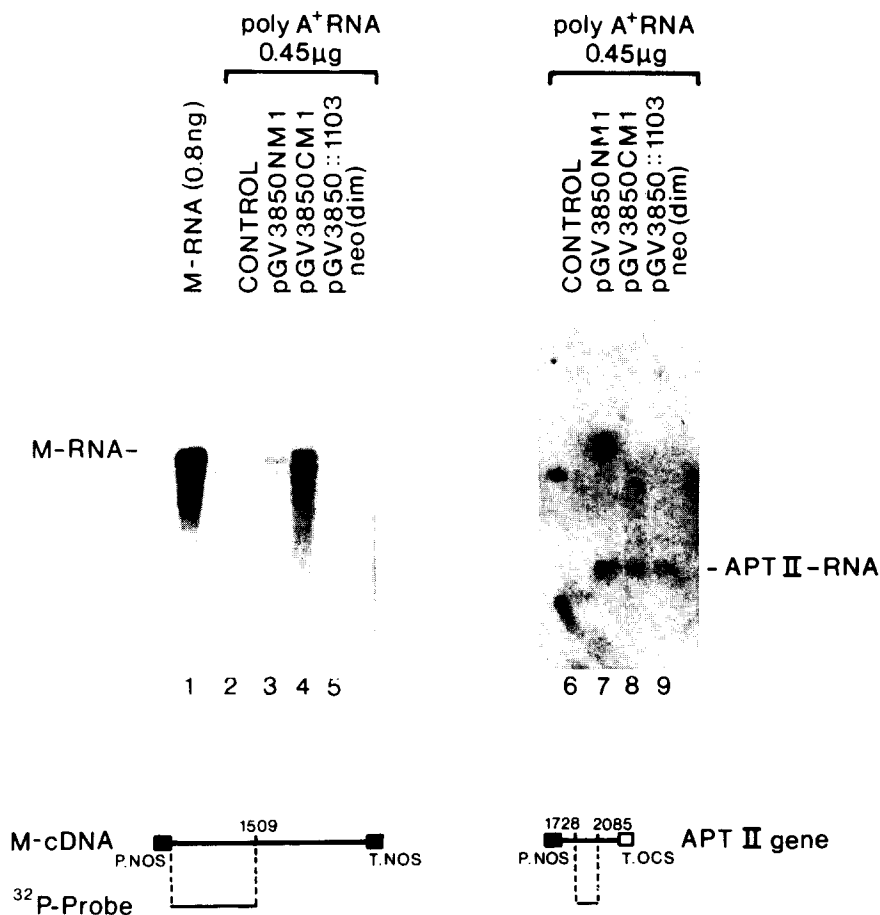


Fig. 5. Northern blot analysis of cowpea callus tissues with integrated DNA copies of CPMV M-RNA. Poly(A)⁺-RNA, isolated from cowpea calli transformed with *A. tumefaciens* carrying plasmid pGV3850NM1, pGV3850CM1, pGV3850 :: 1103neo(dim) or non-transformed (control), was denatured and electrophoresed in a 1% agarose gel containing formaldehyde [18] and hybridized to ³²P-labeled probes. The M13 probe used in panel A contained M-RNA-specific sequences and has been described in Fig. 4A. The M13 probe used in panel B was synthesized from M13KanPS6 which consisted of the 358 bp *Pst*I-*Sph*I fragment from the ATP-II gene of Tn5 [32] inserted into M13mp19 [33]. M-RNA extracted from virions was used as marker. The amounts of RNA used are indicated on top of the figure.

since the CaMV 35S promoter fragment used in the construction of pGV3850CM1 comprised the first 130 bases of the CaMV 5'-leader sequence [17], while at the 3'-end of the transcript another ~200 bases extra were expected originating from the nos terminator fragment. Hence, the full-length transcript of pGV3850CM1-transformed cells

was expected to be approx. 3850 long, i.e. 300 bases longer than natural M-RNA.

The nature of the shorter transcript was not further analyzed. This RNA species could have arisen either by internal start or premature termination of transcription, or, alternatively, by erroneous splicing. Even so, the analysis of Fig. 5 shows that in cowpea

cells, transformed using either pGV3850NM1 or pGV3850CM1, full-length transcripts from the integrated M-RNA copies, comprising up to more than 3500 bases, are detectably produced.

In addition, the results shown in Fig. 5 reveal that in cowpea cells the CaMV 35S promoter is significantly stronger than the nos promoter. While in calli transformed using either pGV3850CM1 or pGV3850NM1, the transcription level of the chimaeric kanamycin genes was similar (cf. lanes 7 and 8 in Fig. 5) the transcription level of the viral cDNA sequence was dependent on the promoter employed. The CaMV 35S promoter appeared to be at least 10-fold stronger in cowpea than the nos promoter (cf. lanes 3 and 4, Fig. 5).

Discussion

Recent reports have pointed out the important applications of integrated DNA copies of plant viral genomes as a valuable tool in studies on the molecular biology of plant viruses, in the development of new types of viral-based vectors and in the identification of cross-protection reactions [26, 27]. The integration and expression of the coat protein gene of TMV in tobacco [17] and the subsequent resistance of transformed plants to TMV has been documented [28] but, in our knowledge, the integration of a full-length DNA copy of a complete viral RNA segment has not yet been described. In this paper we demonstrate that a complete DNA copy of CPMV M-RNA (3481 bases exclusive of the poly(A) tail) can be integrated into the cowpea genome by *Agrobacterium*-mediated gene transfer. Provided with either the CaMV 35S promoter or the nopaline synthase promoter, this copy is efficiently expressed in transformed callus tissue, the first promoter being approximately 10 times stronger than the second one. Full-length transcripts of more than 3500 bases long were obtained, demon-

strating that although natural replication of CPMV RNA occurs in the cytoplasm, long viral RNA can be synthesized in the nucleus and remains stable. At this moment we do not know yet if the RNA transcripts are transported to the cytoplasm. It will be interesting to test if, after superinfection of transformed callus (or protoplasts prepared from it) with purified B component RNA, which codes for the CPMV-specific RNA polymerase [29,30], the M-cDNA transcripts are replicated and progeny virus is recovered. This approach to produce infectious RNA from cloned DNA copies would enable us to produce well defined mutants by modifying such copies on desired places prior to their transfer into cowpea cells.

Another prospect of the chromosomal insertion of the M-RNA copy and its subsequent transcription is to study the translation properties of this RNA in the absence of the viral proteases, which are both encoded by B-RNA [29,31]. Until now, translation products from the integrated M-cDNA copies have not been detected; currently sensitive immunological analyses are being developed to detect viral proteins synthesized in the transformed cells and which could give answers to questions such as whether the two translation initiation sites on M-RNA found to be active upon *in vitro* translation [6,7], are utilized *in vivo*.

Acknowledgement

The authors are grateful to Drs. M. van Montagu and L. Herrera-Estrella for the gift of plasmid pGV3850 :: 1103neo(dim), Dr. M.W. Bevan for the gift of plasmids pNop6 and pCaMV35S, Dr. N. Grimsley for the gift of pCa305, Rijndert de Fluiter for artwork and M^a Teresa Aguado for excellent typing of the manuscript. J.A.G. was supported by an EMBO long-term fellowship.

References

- 1 R. Goldbach and A. Van Kammen, in: J.M. Davies (Ed.), *Molecular Plant Virology*, Vol. II, CRC Press, Boca Raton, 1985, p. 83.
- 2 G. Rezelman, R. Goldbach and A. Van Kammen, *J. Virol.*, 36 (1980) 366.
- 3 R. Goldbach, G. Rezelman and A. Van Kammen, *Nature*, 286 (1980) 297.
- 4 H. Franssen, R. Goldbach, M. Broekhuijsen, M. Moerman and A. Van Kammen, *J. Virol.*, 41 (1982) 8.
- 5 P. Van Wezenbeek, J. Verver, J. Harmsen, P. Vos and A. Van Kammen, *EMBO J.*, 2 (1983) 941.
- 6 H.R.B. Pelham, *Virology*, 96 (1979) 463.
- 7 R.W. Goldbach, J.G. Schilthuis and G. Rezelman, *Biochem. Biophys. Res. Commun.*, 99 (1981) 89.
- 8 P. Vos, J. Verver, P. Van Wezenbeek, A. Van Kammen and R. Goldbach, *EMBO J.*, 3 (1984) 3049.
- 9 J.A. García, J. Hille and R. Goldbach, *Plant Sci.*, 44 (1986) 37.
- 10 H.W. Boyer and D. Roulland-Doussoix, *J. Mol. Biol.*, 41 (1969) 459.
- 11 E. Van Haute, H. Joos, M. Maes, G. Warren, M. Van Montagu and J. Schell, *EMBO J.*, 2 (1983) 411.
- 12 P. Zambryski, H. Joos, C. Genetello, J. Leemans, M. Van Montagu and J. Schell, *EMBO J.*, 2 (1983) 2143.
- 13 A.P. Czernilofsky, R. Hain, L. Herrera-Estrella, H. Lörz, B.J. Baker and J. Schell, *DNA*, (1986) in press.
- 14 F. Bolivar, R.L. Rodriguez, P.J. Greene, M.C. Betlach, H.L. Heyneker, H.W. Boyer, J.H. Crosa and S. Falkow, *Gene*, 2 (1977) 95.
- 15 N. Grimsley, B. Hohn, T. Hohn and R. Walden, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) in press.
- 16 M.W. Bevan, *Nucleic Acids Res.*, 12 (1984) 8711.
- 17 M.W. Bevan, S.E. Mason and P. Goelet, *EMBO J.*, 4 (1985) 1921.
- 18 T. Maniatis, E. Fritsch and J. Sambrook, *Molecular cloning: A Laboratory Manual*, Cold Spring Harbor Lab, NY, 1982.
- 19 G. Ooms, P.J.J. Hooymaas, G. Moolenaar and R.A. Schilperoort, *Gene*, 14 (1981) 33.
- 20 M.G. Murray and W.F. Thomson, *Nucleic Acids Res.*, 19 (1980) 4321.
- 21 L. Weislander, *Anal. Biochem.*, 98 (1979) 305.
- 22 R.B. Horsch, J.E. Fry, N.L. Hoffmann, D. Eichholz, S.G. Rogers and R.T. Fraley, *Science*, 227 (1985) 1229.
- 23 N.S. Shekhawat and A.W. Galston, *Plant Sci. Lett.*, 32 (1983) 43.
- 24 P.S. Thomas, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 5201.
- 25 M. De Block, L. Herrera-Estrella, M. Van Montagu, J. Schell and P. Zambryski, *EMBO J.*, 3 (1984) 1681.
- 26 R.C. Gardner, W.R. Hiatt, D. Facciotti and C.K. Shewmaker, *Plant Molecular Biology Reporter*, 2 (1984).
- 27 H. Bialy and A. Klausner, *Biotechnology*, 4 (1986) 96.
- 28 P.P. Abel, R.S. Nelson, B. De, N. Hoffmann, S.G. Rogers, R.T. Fraley and R.N. Beachy, *Science*, 232 (1986) 738.
- 29 H. Franssen, J. Leunissen, R. Goldbach, G. Lomonosoff and D. Zimmern, *EMBO J.*, 3 (1984) 855.
- 30 L. Dorssers, S. Van der Krol, J. Van der Meer, A. Van Kammen and P. Zabel, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 1951.
- 31 H. Franssen, M. Moerman, G. Rezelman and R. Goldbach, *J. Virol.*, 50 (1984) 183.
- 32 E. Beck, G. Ludwig, E.A. Auerwald, B. Reiss and H. Schaller, *Gene*, 19 (1982) 327.
- 33 J. Norrander, T. Kempe and J. Messing, *Gene*, 26 (1983) 101.